APPENDIX B

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Identification of cold-inducible downstream genes of the *Arabidopsis* DREB1A/CBF3 transcriptional factor using two microarray systems

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Summary

The transcriptional factor DREB/CBF (dehydration-responsive element/C-repeat-binding) specifically interacts with the dehydration-responsive element (DRE)/C-repeat (CRT) *cis*-acting element (A/GCCGAC) and controls the expression of many stress-inducible genes in *Arabidopsis*. Transgenic plants overexpressing DREB1A showed activated expression of many stress-inducible genes and improved tolerance to not only drought, salinity, and freezing but also growth retardation. We searched for downstream genes in transgenic plants overexpressing DREB1A using the full-length cDNA microarray and Affymetrix GeneChip array. We confirmed candidate genes selected by array analyses using RNA gel blot and identified 38 genes as the DREB1A downstream genes, including 20 unreported new downstream genes. Many of the products of these genes were proteins known to function against stress and were probably responsible for the stress tolerance of the transgenic plants. The downstream genes also included genes for protein factors involved in further regulation of signal transduction and gene expression in response to stress. The identified genes were classified into direct downstream genes of DREB1A and the others based on their expression patterns in response to cold stress. We also searched for conserved sequences in the promoter regions of the direct downstream genes and found A/GCCGACNT in their promoter regions from −51 to −450 as a consensus DRE. The recombinant DREB1A protein bound to A/GCCGACNT more efficiently than to A/GCCGACNA/G/C.

Keywords: abiotic stress, transcription factor, downstream genes, stress tolerance, A/GCCGACNT.

Introduction

Plants respond and adapt to environmental stresses such as drought, salinity, and cold with physiological and developmental changes. These stresses induce many plant genes, which function in stress tolerance (for reviews, see Bray, 1997; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997, 2000). The products of these genes are thought to function not only in protecting cells from stress but also in the regulation of genes for signal transduction in stress response (Shinozaki and Yamaguchi-Shinozaki, 2000). Thus, these gene products are classified into two groups. The first group includes proteins that probably function in stress tolerance: water channel proteins, the enzymes required for the biosynthesis of various osmoprotectants, late-embryogenesis-abundant (LEA) proteins that may protect macromolecules and membranes, enzymes for modifying membrane lipids, detoxification

enzymes, and so on. The second group contains protein factors involved in further regulation of signal transduction and gene expression that probably functions in response to stress. Existence of various stress-inducible genes suggests that the response of plants to stress is complex.

Analyses of drought-inducible genes have indicated the existence of ABA-independent and ABA-dependent signal transduction cascades between the initial signal of water deficit and the expression of specific genes (reviewed by Bray, 1997; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 2000). Several stress-inducible genes, such as *rd29A* and *cor15a*, are induced through this ABA-independent pathway. A *cis*-acting element has been identified in the promoter region of *rd29A* and is responsible for drought-, high-salt-, and cold-inducible gene expression. This sequence (TACCGACAT), named the dehydration-responsive element (DRE), is also found in the promoter regions of many drought- and cold-inducible genes (Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 1994). Similar *cis*-acting elements, named C-repeat (CRT) and low-temperature-responsive element (LTRE), both containing an A/GCCGAC core motif, regulate cold-inducible genes (Baker *et al.*, 1994; Jiang *et al.*, 1996; Thomashow, 1999).

cDNAs encoding DRE-binding proteins, CBF1, DREB1A, and DREB2A, have been isolated by the yeast one-hybrid screening method (Liu *et al.*, 1998; Stockinger *et al.*, 1997). The deduced amino acid sequences of these proteins showed significant sequence similarity in the conserved DNA-binding domain found in the ERF and AP2 proteins. These proteins specifically bind to the DRE/CRT sequence and activate the transcription of genes driven by the DRE/CRT sequence in *Arabidopsis*. We isolated two cDNA clones homologous to *DREB1A* (*DREB1B* and *DREB1C*) and one cDNA homologous to *DREB2A* (*DREB2B*; Liu *et al.*, 1998; Shinwari *et al.*, 1998). Gilmour *et al.* (1998) also reported cloning of two *CBF1* homologs, *CBF2* and *CBF3* from *Arabidopsis*. *CBF1* is identical to *DREB1B*, and its two homologs, *CBF2* and *CBF3*, are identical to *DREB1C* and *DREB1A*, respectively. Expression of the *DREB1/CBF* genes is induced by cold stress, and expression of the *DREB2* genes is induced by drought and high salt stresses. DREB1/CBFs are thought to function in cold-responsive gene expression, whereas DREB2s are involved in drought-responsive gene expression.

Strong tolerance to freezing stress was observed in the transgenic *Arabidopsis* plants overexpressing *CBF1* (*DREB1B*) cDNA under control of the cauliflower mosaic virus (CaMV) 35S promoter (Jaglo-Ottosen *et al.*, 1998). We reported that overexpression of the *DREB1A* (*CBF3*) cDNA under the control of the CaMV 35S promoter resulted in strong expression of downstream stress-inducible genes and the transgenic plants had acquired higher tolerance to drought, high salt, and freezing stresses (Gilmour *et al.*, 2000; Kasuga *et al.*, 1999; Liu *et al.*, 1998). However, overexpression of the DREB1A protein also caused severe growth retardation under normal growth conditions. Use of the stress-inducible *rd29A* promoter instead of the constitutive CaMV 35S promoter for overexpressing of DREB1A minimizes negative effects on plant growth (Kasuga *et al.*, 1999). Six genes have been identified as the downstream stress-inducible genes of DREB1A using RNA gel blot analysis. We also identified further downstream stress-inducible genes of DREB1A using 1300 full-length *Arabidopsis* cDNAs microarray (Seki *et al.*, 2001). Twelve stress-inducible genes were identified as downstream genes of DREB1A, and six of them were novel. These DREB1A downstream genes contained the DRE or DRE-related core motifs in their promoter regions. Recently, Fowler and Thomashow (2002) identified 41 downstream genes of CBFs/DREBs using Affymetrix GeneChip arrays.

It is important to identify the downstream genes of DREB in *Arabidopsis* precisely to understand the DRE/DREB (CRT/CBF)-regulated signal pathway and molecular mechanisms of stress tolerance in plants. We searched for the downstream genes of DREB/CBF using the approximately 7000 RIKEN *Arabidopsis* full-length (RAFL) cDNA microarray and the approximately 8000 Affymetrix *Arabidopsis* GeneChip array. We confirmed candidate genes selected by array analyses using RNA gel blot analysis. We identified 38 genes as the downstream genes of DREB1A, 20 of which were unreported new downstream genes. The products of the confirmed downstream genes included not only proteins that function in stress tolerance but also proteins involved in further regulation of signal transduction and gene expression in response to stress. We selected direct downstream genes of DREB1A from the identified downstream genes based on their expression patterns in response to cold stress and existence of the DRE/CRT sequence in their promoter regions. The direct downstream genes of DREB1A/CBF3 satisfy the following three criteria: (i) increased level of transcripts in the 35S:DREB1A transgenic plants under a control condition; (ii) significantly increased transcripts between 2 and 10 h after cold treatment; and (iii) existence of the DRE core motif in the promoter region.

Results

Identification of genes regulated by the DREB1A transcription factor

We have shown that overexpression of the *DREB1A* cDNA under control of the CaMV 35S promoter in transgenic *Arabidopsis* plants (35S:DREB1A transgenic plants) gave rise to strong constitutive expression of the stress-inducible downstream genes and increased stress tolerance to cold,

drought, and high salt stresses. Overexpression of DREB1A also resulted in growth retardation under normal growth conditions. Previously, we identified 13 downstream cold-regulated genes in transgenic plants using RNA gel blot and microarray analyses (Kasuga *et al.*, 1999; Seki *et al.*, 2001; Taji *et al.*, 2002). To determine more downstream cold-regulated genes of DREB1A in *Arabidopsis*, we performed array analysis using the approximately 7000 RAFL cDNA microarray and the approximately 8000 Affymetrix GeneChip array. Preparation of the RAFL cDNA microarray containing the approximately 7000 *Arabidopsis* full-length cDNAs was described previously by Seki *et al.* (2001). A total of 6454 independent *Arabidopsis* genes were included in the RAFL cDNA microarray. The Affymetrix GeneChip array contains 7483 independent *Arabidopsis* genes. We compared genes of the RAFL cDNA microarray and the Affymetrix GeneChip, and found 1919 genes in common (Figure 1). In this study, we examined the expression profiles of 12 018 independent genes, which accounted for almost half of the *Arabidopsis* genes.

The RAFL cDNA microarray analysis was carried out as described before by Seki *et al.* (2001, 2002). We used two independent transgenic *Arabidopsis* plants overexpressing DREB1A, 35S:DREB1Ad, and 35S:DREB1Af. Both transgenic plants showed growth retardation and stress tolerance to drought, high salt, and cold like 35S:DREB1Ab (Kasuga *et al.*, 1999). In both transgenic plants, the *DREB1A* gene was strongly expressed under control condition. mRNAs from the 35S:DREB1Ad and 35S:DREB1Af transgenic plants and wild-type (pBI121) plants were used for the preparation of Cy3- and Cy5-labeled cDNA probes, respectively. These cDNA probes were mixed and hybridized with the cDNA microarray. To assess the reproducibility of the microarray analysis, we repeated the experiment three times. Hybridization of different microarrays with the same mRNA samples indicated a good correlation. We focused on the genes with expression ratios greater than five times in at least either 35S:DREB1Ad or 35S:DREB1Af transgenic plants as compared with the control plants. Analysis with the RAFL cDNA microarray revealed an increase in the level of 19 gene transcripts under the non-stressed normal growth condition in the transgenic plants. Analysis with the Affymetrix GeneChip array was carried out as described by Goda *et al.* (2002). The DREB1A downstream genes were analyzed using the 35S:DREB1Ad and control plants. Signal log ratios were calculated for the duplicate transgenic plant samples compared with each of the two control samples, generating four comparisons. We adopted genes with signal log ratios greater than two times in all four comparisons. Analysis with the Affymetrix GeneChip array revealed an increase in the transcript level of 32 genes under the control condition in transgenic plants.

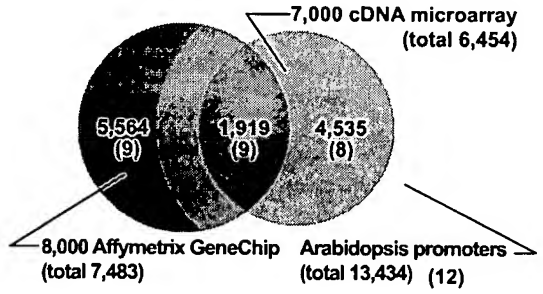

Figure 1. Classification of genes on the RAFL cDNA microarray and the Affymetrix GeneChip array.
A total of 6454 independent *Arabidopsis* genes were included in the approximately 7000 RAFL cDNA microarray. The Affymetrix GeneChip had approximately 8000 DNA oligonucleotide probe sets, and the number of independent *Arabidopsis* genes was 7483. We compared genes of the RAFL cDNA microarray and the Affymetrix GeneChip, and found 1919 genes in common. Therefore, we searched a total of 12 018 genes, which contained almost half of the *Arabidopsis* genes. Seventeen and 18 genes were identified as the downstream genes of DREB1A by the RAFL cDNA microarray and the Affymetrix GeneChip, respectively, and 9 identified genes were in common. We searched 13 434 independent *Arabidopsis* promoters that contain A/GCCGACNT and identified additional 12 genes as downstream genes of DREB1A. The numbers of identified downstream genes are shown in the parentheses.

We selected 19 and 32 genes as candidates for downstream genes of the DREB1A protein using the RAFL cDNA microarray and the Affymetrix GeneChip array, respectively. Among these 51 candidate genes, 9 were upregulated genes found in common by both array analyses. Among the 42 genes, 8 were already reported as downstream genes of DREB1A (Kasuga *et al.*, 1999; Seki *et al.*, 2001; Taji *et al.*, 2002) and 34 candidate genes were unreported at that time and were further analyzed by RNA gel blot hybridization. Twenty-six genes showed increased mRNA accumulation under the control condition in both 35S:DREB1Ad and 35S:DREB1Af transgenic plants as compared with that in wild-type plants (Figure 2a). We confirmed 17 and 18 genes as the downstream genes using RNA gel blot analysis in the 19 and 32 candidate genes selected by the RAFL cDNA microarray and the Affymetrix GeneChip array, respectively. As 9 genes were found in common by both array analyses, 26 genes in total were identified as the downstream genes of DREB1A (Figure 1; Table 1).

The circadian clock has been reported to control expression of the *DREB1A/CBF3* gene and some of the identified downstream genes of DREB1A (Harmer *et al.*, 2000). Therefore, we analyzed the effects of the circadian clock on the expression of DREB1A and its downstream genes in comparison with the effect of cold stress. We analyzed the expression of DREB1A and all identified downstream genes every 3 h for 1 day under a non-stressed control condition and found that all genes except *AtGolS3* and *At2g23120* were induced in the daytime. The expression patterns of DREB1A and the two downstream genes *rd29A* and *cor15A* are shown in Figure 2(b). The level of expression of these genes in the non-stressed condition was less than

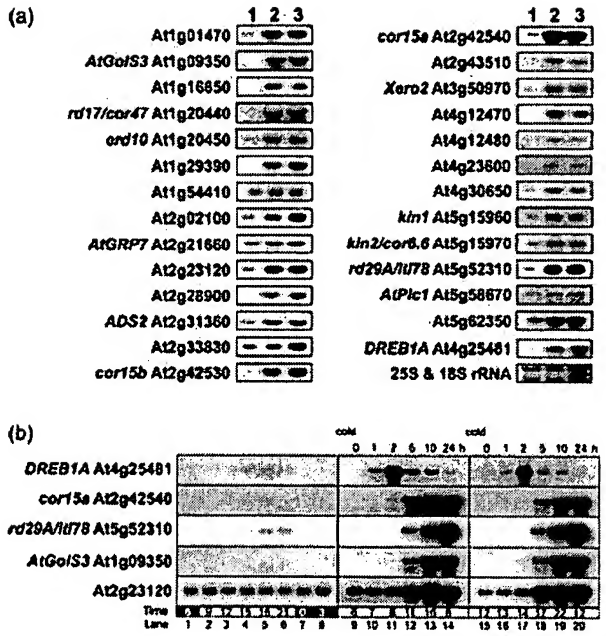

Figure 2. RNA gel-blot analysis of the DREB1A and its downstream genes.
(a) Expression of DREB1A and its downstream genes in the 35S:DREB1A transgenic and wild-type plants. Each lane was loaded with 10 μg of total RNA prepared from the transgenic and wild-type (pBI121) plants. Lane 1 contains RNA prepared from the wild-type plants, lane 2 contains RNA prepared from the 35S:DREB1Ad transgenic plants, and lane 3 contains RNA prepared from the 35S:DREB1Af transgenic plants.
(b) Analysis of effects of the circadian clock on the expression of DREB1A and its downstream genes. Each lane was loaded with 10 μg of total RNA prepared from the wild-type plants. The expression of DREB1A and its downstream genes was analyzed every 3 h for 1 day under the non-stressed control condition (lanes 1–8). Induction of DREB1A and its downstream genes by cold stress was analyzed after starting treatment at two different time points (lanes 9–20).

one-tenth of the maximum induction level under cold stress. Expression of *AtGolS3* was not detected under the non-stressed condition during the 24-h study period (Figure 2b). *At2g23120* was expressed even under the non-stressed condition and the level of expression did not change for 24 h (Figure 2b). Then, we analyzed induction of all downstream genes by cold stress starting treatment at two different time points and found that induction of all *DREB1A* and its downstream genes showed the same expression pattern regardless of starting time (Figure 2b). These results indicate that DREB1A and its downstream genes are clock controlled but that these effects are not important for induction of these genes by cold stress.

Cold regulation of the identified downstream genes of DREB1A

We previously reported that expression of the *DREB1A/CBF3* gene was regulated by cold stress. The expression level of the *DREB1A/CBF3* transcript increased within 1 h after exposure to cold stress (4°C) and reached its maximum at 2 h (Liu *et al.*, 1998; Figures 2b and 3). We verified whether the identified new downstream genes of DREB1A were also regulated by cold stress using RNA gel blot hybridization. As shown in Figure 3, all 26 identified genes were induced by cold stress within 24 h. We compared the expression patterns of these downstream genes in response to cold stress and classified them into three groups: Class I–Class III.

The Class I genes showed markedly increased expression within 5 h and reached a maximum at 10 or 24 h after exposure to cold stress. These Class I genes are indicated in blue in Figure 3. Many reported DREB1A downstream genes, such as *rd29A*, *cor15a*, *cor15b*, *kin1*, *kin2*, *erd10*, *rd17*, and *AtGolS3* belong to this class. The expression of the Class II genes was induced within 2 h and reached a maximum at 5 h after exposure to cold stress. Only one gene belongs to this class and is shown in orange in Figure 3. The Class III genes were induced gradually up to 24 h. Eight genes belong to this class and are shown in green in Figure 3. The expression of the *DREB1A* gene during cold stress preceded that of the Class I genes, which have the DRE core motif in their promoter (except for At4g12470). These results indicated that the Class I genes may be direct downstream genes of DREB1A. On the other hand, the timing of the expression of the Class II and Class III genes is different from that of the Class I genes. These genes probably were regulated by DREB1A through other factors, such as DREB1A-induced transcription factors in *Arabidopsis*, or regulated by both DREB1A and other transcription factors.

Conserved sequence in the promoter region of the downstream genes of DREB1A confirmed by RNA gel blot analysis

In our previous study, the *DREB1A/CBF3* gene encoding DRE-binding protein was isolated by the yeast one-hybrid screening method using *Arabidopsis* cDNA libraries (Liu *et al.*, 1998). The DREB1A protein specifically bound to six nucleotides (A/GCCGAC) of the DRE core motif (Sakuma *et al.*, 2002). We searched for conserved sequences in the promoter regions of the 26 downstream genes of DREB1A confirmed by RNA gel blot analysis using the MEME (Bailey and Elkan, 1995) and SEQUENCE LOGO programs (Schneider and Stephens, 1990). The 1000-bp promoter regions upstream of putative transcriptional initiation sites that are the 5′ ends of the full-length cDNA clones were used for this search (Seki *et al.*, 2002). We found two highly conserved sequences, A/GCCGACNT and ACGTGG/T, that included the DRE core and ABRE (ABA response element) core motifs in these 1000-bp promoter regions (Figure 4a). Among the 26 downstream genes, 18 (69%) and 17 (65%) contained the A/GCCGACNT and ACGTGG/T sequences in their 1000-bp promoter regions, respectively. In addition,

Table 1 List of genes that are upregulated by DREB1A overexpression and cold stress

RAFL clone	AGI	Gene name	Description	Methods	Publication
Transcriptional factor					
RAFL04-15-K19	At1g27730	*STZ ZAT10*	C_2H_2 zinc finger DNA-binding protein	Promoter	New
RAFL21-19-J24	At5g04340		C_2H_2 zinc finger DNA-binding protein	Promoter	New
–	At1g46768	*RAP2.1*	AP2 domain DNA-binding protein	Promoter	4
PI metabolism					
RAFL09-43-P14	At5g58670	*AtPlc1*	Phosphatidylinositol-specific phospholipase	8000	New
RNA-binding protein					
RAFL04-16-B18	At2g21660	*AtGRP7*	Glycine-rich RNA-binding protein	8000	New
Transport protein					
RAFL09-48-J19	At4g35300		Sugar transport protein	Promoter	New
Desaturase					
RAFL05-09-K18	At2g31360	*ADS2*	Delta 9 desaturase	8000	New
Metabolism					
RAFL04-16-K22	At1g09350	*AtGolS3*	Galactinol synthase	7000, 8000	3, 4
RAFL19-51-F16	At4g23600		Tyrosine transaminase	8000	4
RAFL09-23-E17	At4g33070	*Pdc1*	Pyruvate decarboxylase-1	Promoter	New
Protease inhibitor					
RAFL05-08-E12	At2g02100		Gamma-thionins family	7000	New
RAFL05-18-K08	At2g43510		Tryosin inhibitor	8000	New
RAFL11-11-M23	At4g12470		Protease inhibitor/seed storage/LTP family	7000, 8000	4
RAFL06-76-D14	At4g12480		Protease inhibitor/seed storage/LTP family	8000	New
LEA protein					
RAFL05-17-B13	At1g01470		Late embryogenesis abundant protein	7000, 8000, promoter	4
RAFL05-21-F13	At1g16850		Late embryogenesis abundant protein	7000	New
RAFL04-20-N09	At1g20440	*rd17 cor47*	Dehydrin	1300, 7000, 8000	1, 2, 4
RAFL05-08-P17	At1g20450	*erd10*	Dehydrin	1300, 7000, 8000	1, 2, 4
RAFL05-20-N18	At2g42530	*cor15b*	Late embryogenesis abundant protein	7000, 8000, promoter	4
RAFL05-03-A05	At2g42540	*cor15a*	Late embryogenesis abundant protein	1300, 7000	1, 2
RAFL09-19-H05	At3g50970	*Xero2*	Dehydrin	8000, promoter	4
RAFL14-61-K05	At4g15910		Late embryogenesis abundant protein	Promoter	4
KIN protein					
RAFL06-08-N16	At5g15960	*kin1*	Late embryogenesis abundant protein	1300, 7000	1, 2
RAFL04-17-B12	At5g15970	*kin2 cor6.6*	Late embryogenesis abundant protein	1300, 7000	1, 2, 4
Hydrophilic protein					
RAFL04-17-F01	At5g52310	*rd29A lti78*	Hydrophilic protein	1300, 7000, 8000, promoter	1, 2, 4
Senescence-related protein					
RAFL08-19-H17	At2g17840	*erd7*	Unknown protein	Promoter	4
Unknown protein					
RAFL05-18-O20	At1g29390		Unknown protein	7000	New
RAFL04-12-F24	At1g51090		Proline-rich protein	Promoter	New
RAFL06-15-K18	At1g54410		Unknown protein	7000, 8000	New
RAFL04-10-D13	At2g23120		Unknown protein	7000	4
RAFL05-14-K23	At2g28900		Unknown protein	8000	4
RAFL02-02-B06	At2g33830		Unknown protein	7000, 8000	New
RAFL09-65-H03	At4g14000		Unknown protein	Promoter	New
RAFL07-07-J02	At4g24960	*AtHVA22d*	HVA22 family	Promoter	New
RAFL06-07-E01	At4g30650		Unknown protein	7000	New
RAFL03-01-H06	At4g38580	*ATFP6*	Farnesylated protein	Promoter	New
RAFL08-11-P07	At5g17460		Unknown protein	Promoter	New
RAFL04-18-B07	At5g62350		Unknown protein	1300, 8000	2, 4

Publication indicates whether the gene has not been reported as a DREB1A downstream gene before (New) or has already been reported as a DREB1A downstream genes. 1, Kasuga *et al.* (1999); 2, Seki *et al.* (2001); 3, Taji *et al.* (2002); and 4, Fowler and Thomashow (2002).

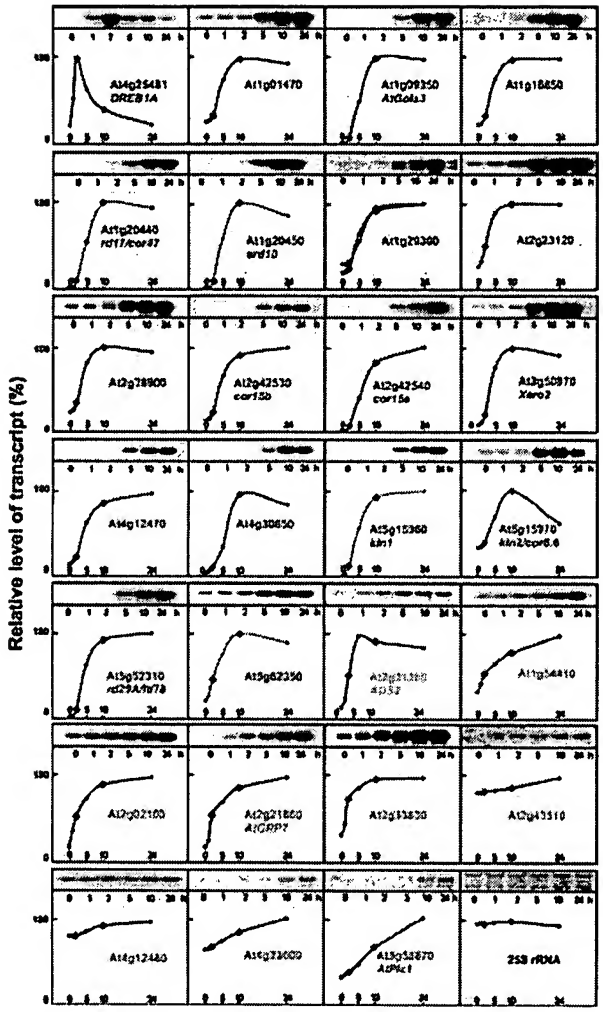

Figure 3. Expression of the DREB1A downstream genes in response to cold stress.

Each lane was loaded with 10 μg of total RNA prepared from *Arabidopsis* plants that had been transferred to and grown for the indicated time at 4°C. The intensity of each band quantified by densitometry shows the levels of expression of the downstream genes. Relative signal values at 2 and 10 h for each gene are indicated by red squares. We compared the expression of these downstream genes in response to cold stress and classified them into Class I–Class III. The Class I genes showed markedly increased expression within 5 h with a maximum at 10 or 24 h, after exposure to cold stress. These Class I genes are indicated by gene names in blue. Expression of the Class II genes was induced within 2 h and reached a maximum at 5 h after exposure to cold stress. One gene belongs to this class and is shown by gene name in orange. The Class III genes were induced gradually during 24 h. Eight genes belong to this class and are shown by gene names in green.

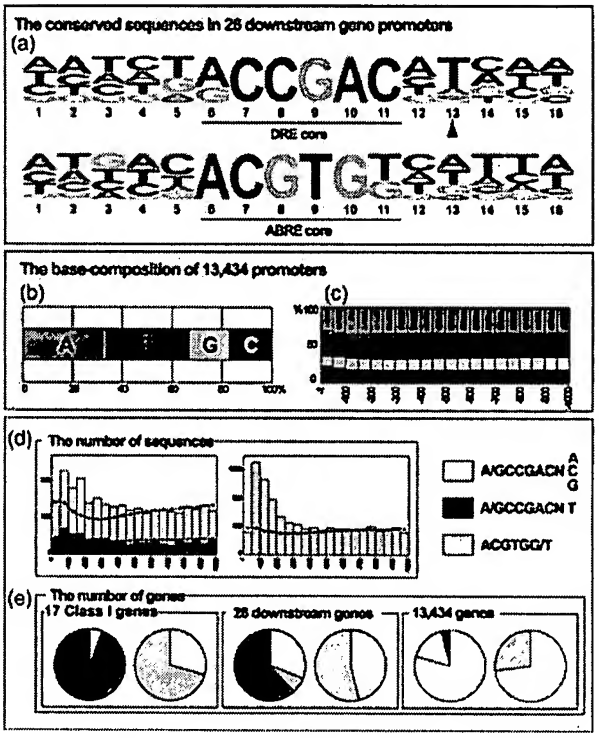

Figure 4. Analysis of DRE and ABRE in the promoter regions of the DREB1A downstream genes or the 13 434 genes in *Arabidopsis*.

(a) Sequence logo for the DRE and ABRE core sequences in the promoter regions of the 26 DREB1A downstream genes. DRE and ABRE core sequences in the promoter regions of the 26 DREB1A downstream genes were used to create the sequence logo. The relative frequencies of bases are given by their relative heights. Statistical significance of the 13th nucleotide T is determined by χ^2 test ($P < 0.001$).

(b) The base composition of the 13 434 *Arabidopsis* promoter sequences.

(c) The base composition of 13 434 *Arabidopsis* promoter sequences in every 50 bp of the 1000-bp promoter sequences. The GC content of *Arabidopsis* promoter is 33.4%.

(d) The number of DRE and ABRE in every 50 bp of the 1000-bp promoter sequences of the 13 434 genes. The expected values of DRE and ABRE are indicated by line charts. Statistical significance was determined by χ^2 test. There are significant differences in frequency of the DRE core and ABRE core motifs in −450 to −51 promoter regions between the found value and the expected value ($P < 0.001$), respectively.

(e) The numbers of the genes having the DRE core and ABRE core motifs in their promoter regions from −450 to −51, are indicated by colored circular charts. The numbers of genes with the A/GCCGAC, A/GCCGACNT, and ACGTGG/T sequences in their promoters are indicated by sky blue, blue, and orange, respectively. DRE and ABRE core elements were found more frequently than their expected number in the promoter regions from −51 to −450 of the 13 434 *Arabidopsis* genes.

Predicted promoter regions of 26 DREB1A downstream genes were compared by the MEME and SEQUENCE LOGO programs. The 1000-bp promoter regions upstream of putative transcriptional initiation sites that are the 5′ ends of the full-length cDNA clones were used for this analysis.

among the 17 Class I genes, 16 (94%) and 14 (82%) contained the A/GCCGACNT and ACGTGG/T sequences in their promoters, respectively.

We estimated the frequency of these conserved sequences in the 1000-bp promoter region in 13 434 independent genes of *Arabidopsis* and compared it with that in the 1000-bp promoter region of the 26 downstream genes. First, we examined the base contents of the 1000-bp promoter sequences in the 13 434 genes and estimated the expected value of these elements. Then, we compared the expected value with the found value of these two elements in the 13 434 promoters. The promoter sequences in the database were AT rich and A:T:G:C was 4 533 367 : 4 407 824 : 2 209 509 : 2 283 300, respectively.

The GC content of the *Arabidopsis* 13 434 promoters was 33.4% (Figure 4b). The expected values of the A/GCCGACNT and ACGTGG/T sequences were 1163 and 6954, respectively. On the other hand, the found values of A/GCCGACNT and ACGTGG/T were 1703 and 9610, respectively. There was no significant difference between the expected value and the found value of the frequency of A/GCCGACNT in the 13 434 independent promoters ($P < 0.05$; χ^2 test). However, the frequency of ACGTGG/T was significantly different between these two values in the 13 434 promoters ($P < 0.001$; χ^2 test). The found value was higher than the expected value of the frequency of A/GCCGACNT.

Then, we divided the 1000-bp promoter sequences into every 50 bp, and searched for the base contents of each 50 bp (Figure 4c). The expected numbers of A/GCCGAC and ACGTGG/T were calculated in the base contents of every 50 bp of the 1000-bp promoter sequences (Figure 4d). We found a hotspot in both DRE core and ABRE core motifs in the promoter region from −51 to −450 bp upstream of putative transcriptional initiation sites of the 13 434 *Arabidopsis* genes (Figure 4d). The found value of the two motifs was significantly higher than the expected value in the promoter region from −51 to −450 bp upstream of the putative transcriptional initiation sites ($P < 0.001$; χ^2 test).

We searched for A/GCCGAC, A/GCCGACNT, and ACGTGG/T in the −51 to −450 promoter region of the 26 downstream genes of DREB1A and the 13 434 independent genes. We found A/GCCGAC, A/GCCGACNT, and ACGTGG/T located in the −51 to −450 promoter regions of 18 (69%), 16 (62%), and 14 (54%) of the downstream genes, respectively (Figure 4e). On the other hand, we found A/GCCGAC, A/GCCGACNT, and ACGTGG/T located in the −51 to −450 promoter region of 2270 (16.9%), 531 (4.0%), and 3674 (27.3%) genes of the 13 434 *Arabidopsis* genes, respectively (Figure 4e). The frequency of these sequences was significantly different between the 26 identified genes and the 13 434 independent genes ($P < 0.001$; χ^2 test). These results indicate that most of the downstream genes have DRE and ABRE in the −51 to −450 promoter region. In addition, the frequency of the A/GCCGACNT sequence in the promoter region of the 26 downstream genes was very high (69%), especially in that of the 17 Class I genes (94%). The A/GCCGACNT sequence may be important for the direct downstream genes of DREB1A.

Competitive DNA binding assay of DREB1A

We performed a gel mobility shift assay with DNA fragments containing the ACCGACAT or ACCGACAA/C/G sequences as competitors to analyze binding affinity of DREB1A. The extent of binding of DREB1A was reduced more effectively by the addition of the unlabeled DNA fragment containing ACCGACAT than by the addition of the unlabeled DNA fragment containing ACCGACAA/C/G

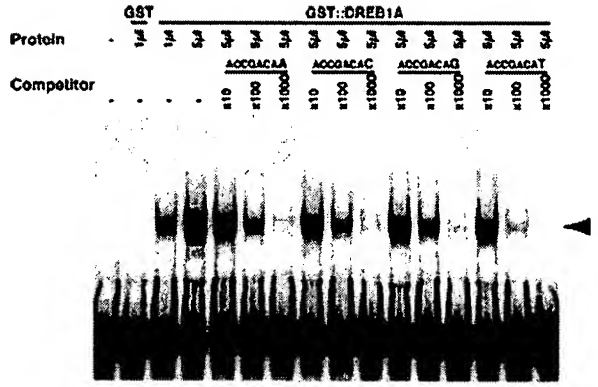

Figure 5. Characterization of the DNA-binding affinities of the recombinant DREB1A proteins.
Sequences of the 75-bp fragment of the wild-type *rd29A* promoter (ACCGACAT) and the mutated fragments (ACCGACAG, ACCGACAA, and ACCGACAC) used as competitors. The ACCGACAT fragment was also used as a probe. Gel mobility shift assay of DREB1A showing a different binding affinity between the wild-type and mutated competitors. GST (glutathion *S*-transferase) or recombinant protein solutions were pre-incubated with or without competitors for 5 min at 25°C. Then, the ^{32}P-labeled probe was added and the mixture was incubated for 30 min at 25°C. As competitors, 10-fold (×10), 100-fold (×100), or 1000-fold (×1000) excess amounts of the unlabeled fragments were used.

(Figure 5). These observations confirmed the results obtained from the analysis of the promoter region of the DREB1A downstream genes (Figure 4). DREB1A binds preferentially to ACCGACAT rather than to ACCGACAA/C/G.

Downstream genes of the DREB1A protein identified by using Arabidopsis *promoter sequence data and RNA gel blot analysis*

As the DRE core motif was found frequently in the promoter region of downstream genes of DREB1A, we searched for *Arabidopsis* genes that contained multi-A/GCCGACNT and -ACGTGG/T to identify other downstream genes. We found 15 genes having multiple elements of both A/GCCGACNT and ACGTGG/T in their promoters. We selected these genes as candidate DREB1A downstream genes. Five of the 15 genes have already been identified as downstream genes. Therefore, we analyzed the expression of the 10 genes in the 35S:DREB1Ad and 35S:DREB1Af plants using RNA gel blot hybridization. Among them, four genes were overexpressed in both transgenic plants under a control condition as compared with the wild-type plants (Figure 6; Table 2).

To analyze the downstream genes of DREB1A comprehensively, we searched further for A/GCCGACNT in the promoter region of cold-regulated genes that were identified using array analyses (Chen *et al.*, 2002; Fowler and Thomashow, 2002; Seki *et al.*, 2002). We selected 30 genes as candidate DREB1A downstream genes and analyzed expression of these genes in the transgenic plants using

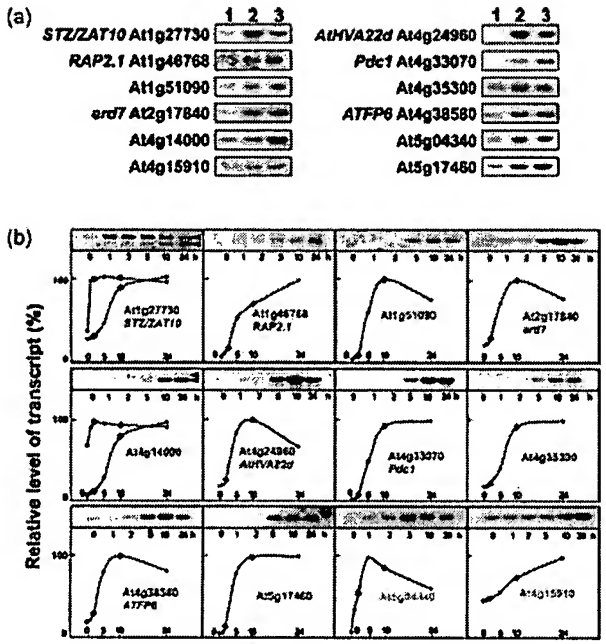

Figure 6. RNA gel-blot analysis of the downstream genes of DREB1A identified by promoter analysis and their expression in response to cold stress.
(a) Expression of the downstream genes of DREB1A in the 35S:DREB1A transgenic and wild-type plants. Each lane was loaded with 10 μg of total RNA prepared from the transgenic and wild-type (pBI121) plants. Lane 1 contains RNA prepared from the wild-type plants, lane 2 contains RNA prepared from the 35S:DREB1Ad transgenic plants, and lane 3 contains RNA prepared from the 35S:DREB1Af transgenic plants.
(b) Each lane was loaded with 10 μg of total RNA prepared from *Arabidopsis* plants that had been transferred to and grown for the indicated time at 4°C. The intensity of each band quantified by densitometry shows the levels of expression of the downstream genes. Relative signal values at 2 and 10 h for each gene are indicated by red squares. We compared the expression of these downstream genes in response to cold stress and classified them into Class I–Class III. The Class I genes showed markedly increased expression within 5 h with a maximum at 10 or 24 h, after exposure to cold stress. These Class I genes are indicated by gene names in blue. Expression of the Class II genes was induced within 2 h and reached a maximum at 5 h after exposure to cold stress. One gene belongs to this Class and is shown by gene names in orange. The Class III genes were induced gradually during the 24 h. One gene belongs to this class and is shown by gene names in green.

RNA gel blot hybridization. Eight genes were overexpressed in transgenic plants under the control condition (Figure 6a; Table 2). We could not detect overexpression of the other 22 genes either in the transgenic plants or the wild-type plants exposed to cold stress (data not shown). Therefore, we identified these additional 12 genes as the downstream genes of DREB1A using the promoter sequence data of *Arabidopsis* genes and RNA gel blot analysis. Twelve additional downstream genes of DREB1A were classified into three groups based on their expression patterns in response to cold stress. Among the 12 genes, 10 belong to Class I, 1 belongs to Class II, and the other 1 belongs to Class III (Figure 6b). A total of 38 genes were identified as the downstream genes of DREB1A and these genes included 20 unreported new downstream genes (Table 1).

Characterization of the downstream genes of DREB1A

We indicated descriptions of the identified genes based on the results of BLAST (Altschul *et al.*, 1990) and PFAM (Bateman *et al.*, 1999) searches in Table 1. These gene products are transcription factors, phospholipase C, RNA-binding protein, sugar transport protein, desaturase, carbohydrate metabolism-related proteins, LEA proteins, KIN (cold-inducible) proteins, osmoprotectant biosynthesis-protein, protease inhibitors, and so on. Twenty-three identified genes have been reported as the DREB1A downstream genes previously and 20 genes were newly identified as the downstream genes in this study. Transcriptional factors contain two kinds of C_2H_2-type zinc-finger DNA-binding proteins. An RNA-binding protein has an RNA-recognition motif containing a glycine-rich region. We have identified genes for many kinds of LEA proteins and protease inhibitors as the downstream genes. The functions of 10 identified genes remain unknown (Table 1).

Discussion

We searched for downstream stress-inducible genes of DREB1A in 35S:DREB1A plants using the RAFL cDNA microarray and the Affymetrix GeneChip array. We selected 19 candidate genes using the RAFL cDNA microarray and confirmed 17 of them as the downstream genes of DREB1A by RNA gel blot analysis. In addition, we selected 32 candidate genes using the Affymetrix GeneChip array, and confirmed 18 of them as the downstream genes of DREB1A by RNA gel blot analysis. The RAFL cDNA microarray contains cDNAs isolated from cDNA libraries prepared from *Arabidopsis* plants exposed to stress such as drought, high salt, and cold stresses. Therefore, most of the stress-inducible genes may be included in the cDNA microarray. To select the downstream stress-inducible genes showing low expression or that are not included in the microarrays, we searched the *Arabidopsis* promoter sequences and identified 12 additional genes as the downstream genes of DREB1A. A total of 38 genes were identified as DREB1A downstream genes, and these genes included 20 new downstream genes. All 38 downstream genes were also upregulated by cold stress in *Arabidopsis* plants (Figures 3 and 6; Kasuga *et al.*, 1999; Seki *et al.*, 2001). Most of the DREB1A downstream genes may be included in these 38 genes.

The downstream genes of DREB1A have already been detected using microarrays by Seki *et al.* (2001) and Fowler and Thomashow (2002), previously. Fowler and Thomashow used the Affymetrix GeneChip and indicated that 41 genes were upregulated in all the CBF1/DREB1B-, CBF2/DREB1C-, and CBF3/DREB1A-overexpressing transgenic

Table 2 List of genes that are found by promoter analysis

RAFL clone	AGI	Gene name	DRE	Position	ABRE	Position
DREB1A downstream genes, which have multiple-DRE and ABRE elements						
RAFL04-12-F24	At1g51090		GTGAC**GCCGAC**ATCAA	−202	AAATG**ACGTGT**ATGAA	−141
			AATGA**GCCGAC**ATGGA	−341		
RAFL04-17-F01*	At5g52310	*rd29A lti78*	CATGG**ACCGAC**TACTA	−275	TTCAT**ACGTGT**CCCTT	−65
			ATCAT**ACCGAC**ATCAG	−225		
			ATACT**ACCGAC**ATGAG	−168		
			ATCAA**GCCGAC**ACAGA	−131		
RAFL05-03-A05*	At2g42540	*cor15a*	TGTTG**GCCGAC**ATACA	−360	ATTAC**ACGTGG**CCTGA	−131
			TCATG**GCCGAC**CTGCT	−183	AGGCC**ACGTGT**AATCA	−128
			AATAA**ACCGAC**AAGGT	−417	CCTTC**ACGTGT**ATTTA	−304
					TTAAT**ACGTGT**AATTT	−70
RAFL05-17-B13*	At1g01470		ATTCC**ACCGAC**GTGCA	−414	ATGTC**ACGTGTT**GATG	−646
			CATCG**ACCGAC**TTCAT	−66	GTTAT**ACGTGT**CTTCT	−147
			AATGG**ACCGAC**CATGT	−384	TGTGT**ACGTGT**GAGGG	−103
RAFL06-13-P08*	At3g24190		GTGGC**GCCGAC**GTAGC	−308	TTGCC**ACGTGG**ACCCT	−100
			TCGTT**GCCGAC**GTAAT	−263		
RAFL07-07-J02	At4g24960	*AtHVA22d*	CTCTCA**CCGAC**CGACG	−335	CCCAC**ACGTGG**CGAGA	−259
			CACCG**ACCGAC**GTCTT	−331	TCGCC**ACGTGT**GGGAT	−262
			CTGAT**GCCGAC**ATACA	−184		
			ATGGC**ACCGAC**CTTAA	−297		
RAFL08-11-P07	At5g17460		GCCAC**GCCGAC**ATAGT	−325	CTGAA**ACGTGT**CTATC	−204
			AACAG**GCCGAC**ATAAT	−139	TAAAC**ACGTGG**CTTAA	−233
					AAGCC**ACGTGT**TTAGT	−236
					ACCCT**ACGTGG**AAACA	−81
RAFL09-19-H05*	At3g50970	*Xero2*	ACTAC**ACCGAC**GTCTT	−237	TCAAT**ACGTGT**TGGCC	−154
			TGTTG**GCCGAC**ATCGT	−162		
			TAGCG**ACCGAC**GTAAT	−254		
RAFL21-19-J24	At5g04340		AAGTA**GCCGAC**TTAAT	−412	CAAAC**ACGTGT**ACCAA	−234
			TCTTA**GCCGAC**TTCCA	−250		
Non-DREB1A downstream genes, which have multiple DRE and ABRE elements						
RAFL05-12-H13	At4g01020		AAGAC**ACCGAC**ATATA	−75	TTGGA**ACGTGT**AAATC	−395
			AATTC**ACCGAC**TTCAT	−404	TAAGC**ACGTGT**GTATA	−196
			GATGA**ACCGAC**AAATC	−112	AGATT**ACGTGT**CTTAG	−136
RAFL06-80-P19			GTTTT**ACCGAC**TTGCT	−51	AAATC**ACGTGT**CCGAA	−81
			GATTT**GCCGAC**TTGGG	−174		
			GATTT**GCCGAC**TTGGG	−87		
RAFL08-12-K03	At2g15320		GCAAA**GCCGAC**ATCCA	−296	TACTA**ACGTGT**TATTG	−455
			GCAAA**GCCGAC**CTCCA	−81	AAAGT**ACGTGG**TGGAG	−65
RAFL14-26-J19	At3g46640		CAAAC**GCCGAC**GGTGA	−852	TTCCA**ACGTGG**ACGAG	−76
			TTACT**ACCGAC**TTAGC	−293	ACTCC**ACGTGG**CTCCA	−240
			TTCAA**ACCGAC**CTAAC	−125	TGATG**ACGTGT**TGAAA	−154
RAFL15-18-E07	At2g39810	*HOS1*	GATTT**GCCGAC**TTGGG	−234	AAATC**ACGTGT**CCGAA	−240
			GATTT**GCCGAC**TTGGG	−147		
			GTTTT**ACCGAC**TTGCT	−270		
			AAATA**GCCGAC**CTGAA	−336		
RAFL16-35-J05	At3g50960		TAGCG**ACCGAC**GTAAT	−166	TCAAT**ACGTGT**TGGCC	−266
			TGTTG**GCCGAC**ATCGT	−258		
			ACTAC**ACCGAC**GTCTT	−183		
DREB1A downstream genes, which are selected by low-temperature and DRE elements						
RAFL03-01-H06	At4g38580	*ATFP6*	GTCAC**ACCGAC**GTACC	−480		
			GACGT**ACCGAC**CTTGA	−488		
RAFL04-15-K19	At1g27730		TTATA**GCCGAC**CTCTT	−285	CCGAA**ACGTGT**ACCAT	−400
					CACAC**ACGTGT**ACTAG	−240

Table 2 continued

RAFL clone	AGI	Gene name	DRE	Position	ABRE	Position
RAFL08-19-H17	At2g17840	*erd7*	AAGCG**ACCGAC**CGACA	−210	TTTTA**ACGTGG**CAATC	−989
			GACCG**ACCGAC**ATGAG	−206	GGAAG**ACGTGG**ATGTT	−92
RAFL09-23-E17	At4g33070	*Pdc1*	TTTAG**ACCGAC**ATAAA	−187	GTTAT**ACGTGG**GAACA	−339
					GATCT**ACGTGT**ATCTT	−694
RAFL09-48-J19	At4g35300		ATTAT**GCCGAC**ATTAA	−389		
RAFL09-65-H03	At4g14000		CACAG**ACCGAC**TTTAA	−994		
			CTCGT**ACCGAC**CGGTT	−62		
			TGGAA**GCCGAC**TAAAA	−658		
			CGGAA**GCCGAC**CAAAG	−571		
			TTTCT**ACCGAC**TGAAT	−398		
RAFL14-61-K05	At4g15910		ATCTC**ACCGAC**CTCTT	−388	CTAGAA**CGTGT**CACTT	−413
RAP2.1	At1g46768		CTATG**GCCGAC**ATAAA	−44		
			AGGAG**ACCGAC**TGAAA	−149		

*The genes with an asterisk mark are identified in array analysis.

plants and 30 of them were cold-upregulated genes. Only 11 of the 41 genes identified by Fowler and Thomashow were identical to those of the 18 downstream genes that we identified by Affymetrix GeneChip array analysis. This discrepancy may have been caused by the difference in the annotation table used for Affymetrix GeneChip array analysis. The annotation tables of the Affymetrix GeneChip array have been frequently updated. We used the TAIR (The Arabidopsis Information Resource) annotation table updated in December 2002. In addition, we used the *Arabidopsis thaliana* ecotype Columbia, but they used *A. thaliana* ecotype Wassilewskija-2. Different stress-inducible downstream genes of DREB1A may function in these different ecotypes. However, it may be difficult to determine the downstream genes accurately using only array analysis with repetition experiments. Therefore, we confirmed the downstream genes of DREB1A using RNA gel blot analysis. Only 18 genes were confirmed as DREB1A downstream genes by RNA gel blot analysis among 32 genes identified by the Affymetrix GeneChip.

The direct downstream genes for DREB1A/CBF3 satisfied the following three criteria after RNA gel blot analysis: (i) increased level of transcripts in the 35S:DREB1A transgenic plants under a control condition; (ii) significantly increased transcripts between 2 and 10 h after cold treatment; and (iii) existence of the DRE core motif in the promoter region. The expression of the direct downstream gene was increased markedly after 2 h of cold treatment as the expression of the *DREB1A/CBF3* transcript reaches its maximum at 2 h after a temperature shift from 22 to 4°C (Liu *et al.*, 1998; Figure 2b). In the present study, expression levels of the Class I genes were increased markedly within 5 h and reached their maximum at 10 or 24 h after exposure to cold stress (Figure 3). All the Class I genes except At4g12470 contained a conserved sequence (A/GCCGACNT) as a DRE core sequence in their −51 to −450 promoter regions, while most of the Class II and Class III downstream genes did not contain this sequence in their promoter regions. These results indicate that the Class I genes excluding At4g12470 are the direct downstream genes of DREB1A.

To investigate the importance of A/GCCGACNT as a DRE consensus sequence, we searched for the genes that have multiple A/GCCGACNT and ACGTGG/T in the −51 to −450 promoter region. We selected 15 candidate genes in *Arabidopsis* (Table 2). Nine of them were upregulated under the control condition in the 35S:DREB1A transgenic plants, and were shown to be upregulated by cold stress using RNA gel blot analysis (Figure 6). However, the other six genes were not overexpressed in the transgenic plants (data not shown). In addition, we performed another search to examine the role of A/GCCGACNT on cold-inducible gene expression regulated by DREB1A. We searched for the genes containing A/GCCGACNT in the −51 to −450 promoter region that were upregulated by cold stress. The cold-inducible genes were selected using array analysis data demonstrated by Seki *et al.* (2001, 2002), Fowler and Thomashow (2002), and Chen *et al.* (2002). We selected 30 candidate genes in *Arabidopsis*, and analyzed the expression of these genes under a cold stress condition using RNA gel blot analysis. Eight of them were overexpressed in the 35S:DREB1A plants and upregulated by cold stress using RNA gel blot analysis (Table 2; Figure 6). However, we could not detect the overexpression of 22 genes either in the 35S:DREB1A plants or in the wild-type plants exposed to cold stress (data not shown). Furthermore, we found that 531 genes contain A/GCCGACNT in their promoter regions from −450 to −51 in the 13 434 *Arabidopsis* genes. Many of these genes were not influenced by DREB1A, although they had A/GCCGACNT. These results indicate that A/GCCGACNT in −450 to −51 promoter region

is an efficient *cis*-elements for the expression of genes regulated by DREB1A but another element, such as the other specific sequences or some secondary structure of the promoters, in the promoter region may be necessary for the expression of these genes.

We investigated the functions of the identified genes (Table 1), and estimated how overexpression of DREB1A in the transgenic plants improved stress tolerance. The identified downstream genes of DREB1A were classified into two groups. The first group includes proteins that probably function in stress tolerance such as LEA proteins, antifreeze proteins, hydrophilic protein, RNA-binding protein, an enzyme required for biosynthesis of sugars (galactinol synthase; Taji *et al.*, 2002), and protease inhibitors. These gene products probably function to increase the tolerance to drought, high salt, and freezing stresses in transgenic *Arabidopsis*.

The second group contains protein factors involved in further regulation of signal transduction and gene expression that probably functions in response to stress. They are transcription factors (C_2H_2 zinc finger DNA-binding proteins and ERF/AP2-type DNA-binding protein) and enzymes in phospholipid metabolism (phospholipase C). The genes that encode the transcriptional factors containing C_2H_2-type zinc-finger motif constitute a large family in higher plants (Takatsuji, 1999). The transcription factors STZ and At5g04340 are such DREB1A downstream genes. STZ has been shown to repress the *trans*-activation of genes through an essential DLN-box/EAR-like repression motif in its C-terminal region (Ohta *et al.*, 2001). The At5g04340 protein also has this repression motif in its C-terminal region. Recently, we analyzed transgenic *Arabidopsis* plants overexpressing STZ using a cDNA microarray and found that many genes related to photosynthesis and carbohydrate metabolism were suppressed in these plants (unpublished results by Sakamoto *et al.*). These results suggest that STZ is involved in the mechanism of growth retardation of the 35S:DREB1A transgenic plants. Stress-related signaling has been reported to result in the repression of genes associated with plant growth and development (Logemann *et al.*, 1995). Thus, DREB1A controls not only activation of genes involved in stress tolerance but also repression of genes involved in plant growth and development, such as photosynthesis-related genes. The wide range of functions of the downstream genes of DREB1A indicates that DREB1A regulates a complex gene expression network in response to cold stress.

Experimental procedures

Plant materials and cold stress treatments

Arabidopsis thaliana (ecotype Columbia) and the transgenic 35S:DREB1A plants (Kasuga *et al.*, 1999) were grown under conditions of 16 h light/8 h darkness at 22°C on germination medium agar plates for 3 weeks. Cold stress treatment was performed as described before by Yamaguchi-Shinozaki and Shinozaki (1994).

Microarray analysis

Total RNA was prepared using TRIZOL Reagent (Life Technologies, Rockville, MD, USA) from whole *Arabidopsis* plants, and mRNA was prepared using the mRNA isolation kit (Miltenyi Biotec, CA, USA), as described by Seki *et al.* (2001). For the RAFL cDNA microarray analysis, each mRNA sample was reverse transcribed in the presence of Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia, Piscataway, NJ, USA). Hybridization and scanning analysis were carried out as described before (Seki *et al.*, 2002). For the analysis using the Affymetrix GeneChip array, biotin-labeled cRNA probe sets were generated from the double-stranded cDNAs using the BioArray High Yield RNA transcript labeling kit (Enzo Diagnostics, NY, USA). Hybridization and scanning analysis were carried out as described before by Goda *et al.* (2002).

Data analysis

Image analysis and signal quantification were performed with QUANTARRAY version 2.0 (GSI Lumonics, Oxnard, CA, USA). Background fluorescence was calculated on the basis of the fluorescence signal of the negative control genes (the mouse nicotinic acetylcholine receptor epsilon-subunit (nAChRE) gene and the mouse glucocorticoid receptor homolog gene). A control template DNA fragment (TX803; Takara, Kyoto, Japan) was used as an external control to equalize hybridization signals generated from different samples. Gene-clustering analysis was performed with GENESPRING 5.0 (Silicon Genetics, San Carlos, CA, USA).

A consensus motif search of promoter regions was performed at the Multiple Em for Motif Elicitation (http://meme.sdsc.edu/meme/website/meme.html). Analysis of consensus motifs was performed with DNASIS PRO version 2.0 (Hitachi Software Engineering, Tokyo, Japan). Statistical significance was determined by χ^2 test. The sources of DNA sequences in promoter regions were used at the RIKEN Arabidopsis Genome Encyclopedia (http://rarge.gsc.riken.go.jp/).

RNA gel blot analysis

The RAFL cDNA probes whose sequences were determined by the *Arabidopsis* subspecies (SSP) sequencing consortium and RIKEN Genomic Sciences Center were amplified by polymerase chain reaction (PCR). RNA gel blot hybridization was performed as described before by Liu *et al.* (1998). The RAFL cDNAs are available from RIKEN Bioresource Center.

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